

DRY HEAT DESTRUCTION RATES OF BACILLUS SUBTILIS VAR. NIGER IN A CLOSED SYSTEM

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INTRODUCTION

During the past six months we have begun a study of dry heat destruction rates of spores in a closed system. To date we have developed one type of apparatus for use in this project and have obtained some preliminary data.

OBJECTIVE

The objective of this project is to determine dry heat D-values of microorganisms in closed systems: specifically it is to determine 1) the relationship of spore D-value to spore water content, 2) the effect of the atmospheric volume per spore on the D-value of spores with different initial water contents 3) the effect of pressure on D-value, 4) the effect of water absorption and water vapor transfer characteristics of plastic materials on the D-values of microorganisms adjacent to or imbedded in these materials, and 5) if there are differences between the D-values of spores encapsulated in metals vs. spores encapsulated in plastics.

LITERATURE REVIEW AND BACKGROUND

The most fundamental need of both NASA and the scientific community is to know the precise relationship of water and spore D-value. The problem is severely complicated by two factors: 1) it is nearly impossible to measure the quantity of water in the spore and 2) it is extremely difficult to keep the water in the spores constant.

It is apparent at this time that the spore D-value is very sensitive to minute changes in water content. Starting with spores at ambient conditions of 35% RH at 22°C and heating to 100 or 125°C in this atmosphere (35% RH at 22°C) will result in a loss of water from

spores on surfaces and a decrease in D-value. Encapsulated microorganisms may also lose water; however, their water loss will depend on the surrounding physical system. If a device or system is assembled under normal ambient water vapor conditions (35% RH at 22°C) the encapsulated microorganisms will have a larger D-value than they would have under any lower water condition. Therefore any drying of the system will result in a smaller D-value.

One of the critical locations of encapsulated microorganisms is in and under potting compounds or conformal coatings. At the present time the $D_{125^{\circ}\text{C}}$ for encapsulated microbial spores is five hours. This value was obtained from laboratory experiments where Bacillus subtilis spores were encapsulated in Lucite and epoxy rods and the rods were placed in sealed thermal-death-time tubes to be heated in an oil bath.¹ The conditions to which those spores were subjected will certainly be similar to the conditions to which spores encapsulated in large pieces of Lucite or epoxy will be subject to on the spacecraft. However, were the conditions used by Angelotti the same as the conditions of spores located under thin layers (less than 0.5 inches) of plastic? Since the sealed tube retained the water vapor from the plastic rod it is possible that the D-value of the spores in the rod under these conditions was higher than if the rod were heated outside the glass tube. We believe that the potential gain from a reduction in D-value is sufficient to warrant experiments in this area.

In the sterilization cycles for spacecraft hardware the come-up time for the hardware before there is a significant lethal effect may be five to ten hours. During this time water may be lost from both the plastic rod and the encapsulated spores; this will result in a reduced spore D-value.

Encapsulated spores were studied extensively in the early part of the NASA Planetary Quarantine Program. Most of the studies of encapsulated spores were carried out when there was little understanding of the critical role played by water in the dry heat system. The present research has been designed to start where earlier studies ended, using the new

¹Angelotti, et. al. "Influence of Spore Moisture Content on the Dry-Heat Resistance of Bacillus subtilis var. niger," 16 Appl. Microbiol. 735-745 (1968)

knowledge of dry heat spore destruction to design experiments that will hopefully give a more complete picture of the dry heat destruction characteristics of encapsulated microorganisms.

Development and Use of Cylindrical Heat Block Apparatus

The cylindrical heat block apparatus has been developed to make it possible to study dry heat destruction under a wide variety of closed system conditions. The heat block apparatus consists of a top and bottom unit held together with Allen cap screws. The units are made out of type 303 stainless steel. The bottom unit (Figure 4.1) contains the DASH No. 222 O-ring groove and a 1.275" diameter by 0.020" recess to hold the cylindrical sample disc. To date three types of top units (Figure 4.2) have been constructed: a flat unit, a unit with a 1.185" x 0.25" recess and a unit with a 1.185" x 0.50" recess.

Auxiliary devices required when using the heat-block apparatus are: a torque wrench (Craftsman No. 12-9G44441) for uniformly tightening the cap screw (Figure 4.3), a magnetic wand for removing the sample carrying discs from the recess in the bottom unit (Figure 4.4) and a rack with a handle for holding the block when it is placed in the oil bath (Figure 4.5).

Heating has been carried out using a model MW-1155C-1 "Magni-Whirl" (Blue M Electric Co.) oil bath filled with "U con" HTF-100 heat transfer fluid. A large stainless steel container filled with ice cubes and tap water is used to cool the heat block after heating.

Temperatures in the oil bath are monitored during testing using a model 15305836 Honeywell temperature-recording potentiometer. The temperature is sensed by bare 24-gauge copper constantan thermocouples.

Evaluation of Heating and Cooling Time Lags

The considerable mass of the heat blocks plus the fact that they are made out of stainless steel means that they will not instantly heat and cool. The heating and cooling characteristics of a heat block with a flat top have been evaluated. Heating studies were made both with a thermocouple soldered to the spore-sample disc and with the thermocouple in the air in recess. A lag correction factor of 12.5 minutes was

calculated. Details of the heating and cooling time lag study will be included in the next progress report.

Test Procedure

The Bacillus subtilis var. niger spore crop No. AAAB (see spore code details, Appendix B) was used in all experiments. The stock spore suspension was resuspended in distilled water so that an 0.02 ml droplet would contain 5×10^5 spores. The spores were deposited on the discs using an 0.02 ml push-button (Eppendorf) pipette.

Stainless steel discs were punched out of type 302 full-hard stainless steel shim stock. The discs were thoroughly cleaned. As many as ten discs were placed on a sterile stainless steel sheet in a clean room for inoculation. Four 0.02 ml spore suspension deposits were placed on each disc. Before starting the pipetting operation the spore suspension was mixed for a few seconds using a vibrating automatic mixer (Vortex) and was remixed after each ten pipettings. The inoculating scheme order is shown in Figure 4.6. The discs were randomly assigned to heating times. Both the strips and the blocks with O-rings were allowed to equilibrate in the clean room at 35% RH at 22°C for 18 to 24 hours before assembly.

The blocks were assembled by placing an inoculated disc, with a sterile disc on top of it, into the recess of the bottom block. The screws were then inserted and tightened and the sealed block was immersed into the constant-temperature ($125^\circ\text{C} \pm 1^\circ\text{C}$) oil bath for the specified length of time. At the end of heating, the blocks were cooled for two minutes in the 0°C ice bath and were then wiped dry. The dry block was inserted into the block holder, the screws were removed and the top unit was aseptically removed. The magnetic wand was chemically sterilized using peracetic acid and was then used to remove the two discs. Using a sterile forceps to hold the inoculated disc, it was cut into four pieces (one spore inoculum per piece) using a metal-cutting bench shears (Whitney-Jensen No. 39) that was also sterilized in peracetic acid.

Each stainless steel piece was assayed individually according to NASA Standard Procedures for the Microbiological Examination of Space Hardware, October, 1968.

The data were analyzed to determine the D-value, γ -intercept and the respective confidence intervals.

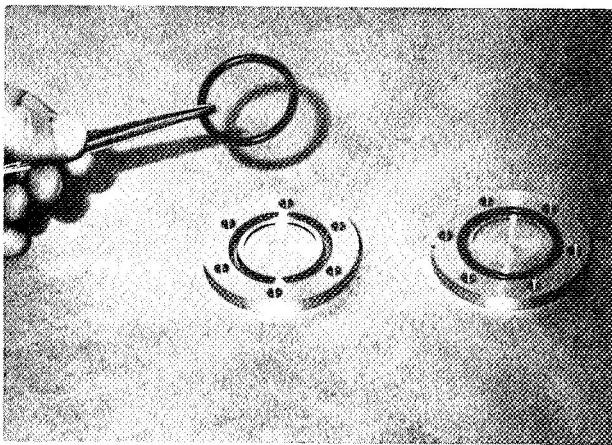


Figure 4.1 - Bottom unit of heat block system and O-ring

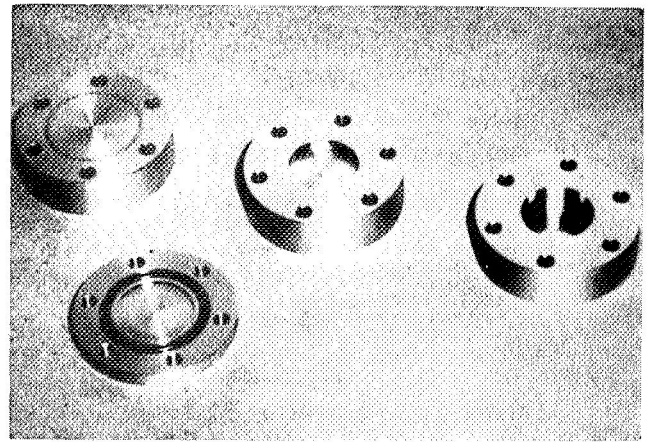


Figure 4.2 - Three types of top units with one bottom unit

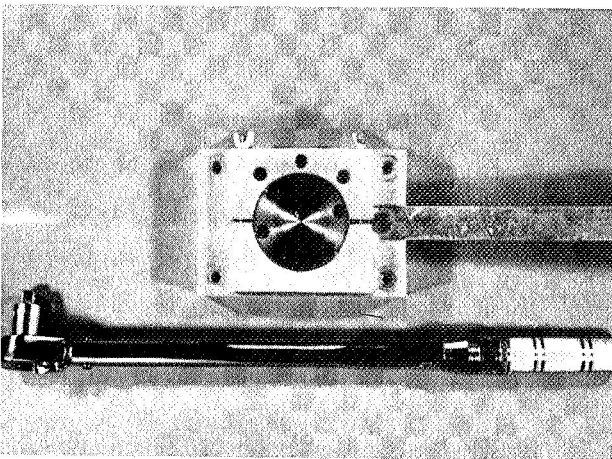


Figure 4.3 - Torque wrench and block-holding apparatus

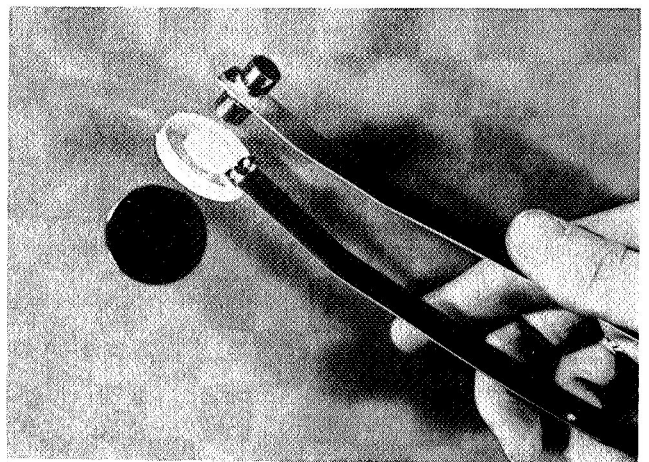


Figure 4.4 - Magnetic wand for removing the sample discs from the recess in the bottom block

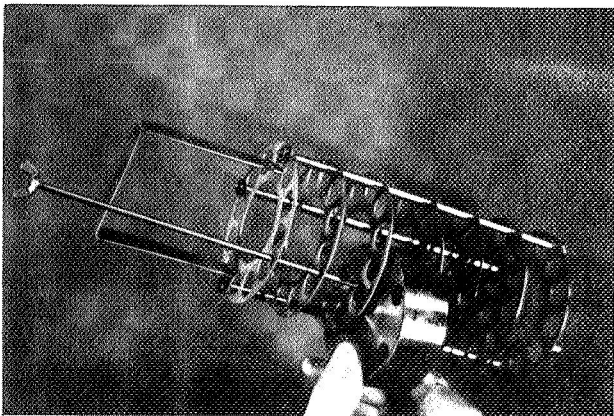


Figure 4.5 - Rack for supporting block during handling in and out of the oil bath

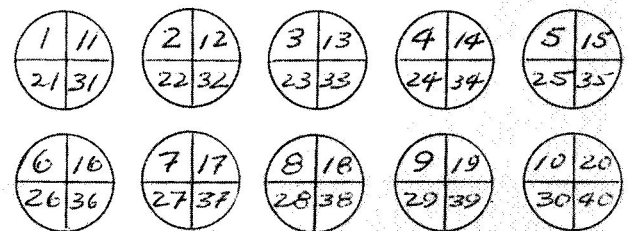


Figure 4.6 - The inoculating-scheme order for the discs

RESULTS AND DISCUSSION

The results of the two sets of replicated tests completed to date are summarized in Table 4.1. Five tests were carried out at 35% RH at 22°C using the Teflon O-rings and for three tests the Viton² O-rings were used.

Table 4.1 - Preliminary D-value Results Showing the Effect of Two Kinds of O-rings Using the Cylindrical Heat Block System with a Flat Top Unit with Two .010-inch-thick Discs in Bottom Unit Recess

<u>O-ring Material</u>	<u>Equilibration Condition</u>	<u>D₁₂₅ min.</u>
Teflon	Clean Room 40% RH at 23°C	27-42
Viton	Clean Room 35% RH at 23°C	80-91

Teflon O-rings were initially chosen as the block seal because it was decided that the O-water absorbing characteristics and heat resistance of Teflon were necessary requirements for this study at 125°C. Unfortunately, Teflon becomes amorphous at 121°C and loses its resiliency. This property of Teflon probably permitted periodic leakage. After deciding that it was possible that the Teflon allowed water vapor to escape, Viton O-rings were chosen because of their good sealing characteristics at high temperatures (up to 204°C) and their low water absorbing properties.

The increase in the D-value of the tests where Viton O-rings were used as compared to the tests using Teflon is difficult to explain on any other than a pressure and water vapor-loss basis.

The heat block system that has been developed appears to perform very well and will allow us to proceed to carry out experiments to answer the several questions regarding the dry heat destruction rates of micro-organisms in closed systems.

²Viton - a synthetic rubber able to withstand a temperature of 200°C in contact with most oils, chemicals, solvents, and fuels. Has good mechanical properties, also resistance to ozone, oxygen, and weathering. Commercial development: DuPont. The Merck Index. 7th edition. (Rathway, New Jersey: Merck and Co. Inc., 1960).

FUTURE WORK

In the immediate future, we will attempt to relate the quantity of water in and surrounding the spore to dry heat destruction characteristics. Initially we will use Bacillus subtilis var. niger spores. However, we will ultimately want these data for several species of microorganism.

In the first series of studies, those spores on stainless steel discs will be equilibrated at several relative humidities ($\approx 0, 20, 35, 50$, and 75%) and will be carried out at 125°C with subsequent tests at other temperatures so that Z-value data can be obtained. This series will be completed by evaluating at least one other species of microbial spore.

In the second series of tests we plan to relate variation in initial water to variation in the volume of atmosphere per spore. Initial studies will be carried out at 125°C using Bacillus subtilis var. niger and at least one other species will also be studied.

A series of experiments will be carried out to evaluate the effect of total gas pressure during heating on the D-value as a function of spore water-content, temperature, and microbial species.

The effect of plastic materials in contact with spores during heating will be studied in a series of experiments. The variables will be: type of plastic, initial water content of plastic, initial spore water-content, temperature, and species of microorganism.